Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com

Efficient direct shoot organogenesis, genetic stability and secondary metabolite production of micropropagated *Digitalis purpurea* L.

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ARTICLE INFO

Keywords:
Cardenolides
Direct organogenesis
Foxglove
Genetic stability
Quality specification

ABSTRACT

Cardiovascular and cancer diseases are the first causes of death in the world. *Digitalis purpurea* L. is a medicinal plant that produces secondary metabolites, like digoxin and digitoxin, which are employed in therapies against heart failure. Moreover, anticancer and antiviral properties of these metabolites have recently been described. The present work details a method to obtain *in vitro* plants of *D. purpurea* from leaf segments through direct organogenesis. A reliable and efficient plant induction system was established by optimizing the concentration of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (6-BAP). The highest frequency of shoot regeneration (98.5%) with an average number of shoots per leaf segment of 18.9 was achieved *via* direct organogenesis from leaf segment on MS medium containing 0.54 μM NAA + 13.2 μM 6-BAP. Additionally, Random Amplified Polymorphic DNA (RAPD) analysis showed 100% monomorphic bands, which indicated genetic stability of the obtained plants. Moreover, leaf powder of de novo regenerated plants fulfills the quality specifications of the British Pharmacopoeia and HPLC analysis revealed the presence of digoxin (22.6 μg gDW^{-1}) and digitoxin (220.7 μg gDW^{-1}) without significant differences in contents between de novo regenerated and mother plants. An efficient *in vitro* propagation protocol *via* direct organogenesis and genetic stability assessment of *D. purpurea* for obtaining leaf powder with quality for the use as raw material have thus been described. The protocol also provides an effective means for several approaches in biotechnology and breeding programs, in order to produce pharmaceutically interesting cardenolides.

1. Introduction

Digitalis purpurea L. is one of the most important medicinal plants, which has been used for many years. In 1785 the British physician William Withering described its pharmacological properties which make it very useful for the treatment of different cardiovascular diseases (Verma et al., 2016). *D. purpurea* plants produce cardenolides, mainly digoxin and digitoxin, which are a group of remarkable chemical compounds that are responsible for these pharmaceutical activities (Agrawal et al., 2012). Several pharmacological applications of these compounds have been reported, for instance, in chronic auricular fibrillation and cardiac insufficiency (Feussner and Feussner, 2010). For these purposes digoxin is the most used cardenolide, with total worldwide sales of US$ 142 million in 2012 (IARC Working Group, 2016).

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https://doi.org/10.1016/j.indcrop.2018.02.067
Received 11 September 2017; Received in revised form 30 January 2018; Accepted 22 February 2018
Available online xxx
0926-6690/

Moreover, antiproliferative and apoptotic effects were observed in several cancer cell lines (Rocha et al., 2014). These effects are related with the inhibition of the Na^+/K^+ ATPase that results in the later activation of the Na^+/Ca^{2+} pump, the increase of intracellular concentration of Ca^{2+} and the induction of apoptosis in cancer cells (reviewed in Elbaz et al., 2012). Nevertheless, other antiproliferative mechanisms have been proposed and are under investigation (Elbaz et al., 2012; Wei et al., 2013; Lin et al., 2015). Recently, antiviral properties against several human-infecting viruses like HIV, herpes, cytomegalovirus and adenovirus have been attributed to cardenolides (Bertol et al., 2011; Cai et al., 2014; Grosso et al., 2017; Zhyvoloup et al., 2017). In the case of HIV digoxin repressed viral gene expression by targeting the cellular Na^+/K^+ ATPase (Zhyvoloup et al., 2017). However, the use of *D. purpurea* for pharmacological purposes needs some further research.

Currently, the only source of cardenolides is the plant itself. Chemical synthesis of these compounds is unviable right now, due to their structural complexity (Verma et al., 2016). Nevertheless, multiple factors modulate cardenolide concentration in plants cultivated in the field, *e.g.* temperature, mineral soil composition, season, humidity and others (Sales et al., 2011). Furthermore, wild populations of *Digitalis* species are significantly affected by large-scale and uncontrolled exploitation in order to satisfy the pharmaceutical industry (Verma et al., 2016). Another important issue of *Digitalis* cultivation is the low germination rate of the seeds. In addition, there are some regions where the plant cannot be grown in open fields, like Cuba (basically because of the high temperature and humidity). As a consequence, several research groups have developed biotechnological strategies in order to reduce the excessive use of natural *Digitalis* populations, to conserve high yielding cardenolide producing plants or for genetic improvement. Such strategies include somatic embryogenesis (Lindemann and Luckner, 1997), temporary immersion systems (Pérez-Alonso et al., 2009, 2012), precursors addition, elicitation (Pérez-Alonso et al., 2014a; Patil et al., 2013) and organogenesis (Hagimori et al., 1982; Pérez-Bermúdez et al., 1984; Cacho et al., 1991; Fatima et al., 2009; Çördük and Aki, 2010; Gurel et al., 2011; Verma et al., 2011a,b; Karimi and Kazemitabar, 2013; Li et al., 2014; Pérez-Alonso et al., 2014b; Yücesan et al., 2014; Kreis et al., 2015; Mohammed et al., 2015). Organogenesis can be done directly or indirectly, direct organogenesis being the most successful for many species of the genus *Digitalis*. This morphogenetic process of plant regeneration allows the generation of entire plants, in a very easy, rapid, homogenized and continuous way during the whole year, without environmental restrictions.

Nevertheless, the scientific literature revealed the application of direct regeneration for *in vitro* production of *D. purpurea* only in studies by Patil et al. (2013) and Li et al. (2014). The latter protocol was used for *Agrobacterium tumefaciens*-mediated genetic transformation. However, neither analysis of leaf powder quality nor genetic fidelity of *in vitro* plantlets was assessed by these authors.

The aim of this research was to carry out efficient *in vitro* plant regeneration of *Digitalis purpurea* L. *via* direct organogenesis and to evaluate the genetic stability of the de novo regenerated plants, in order to obtain metabolites with pharmaceutical quality specifications.

2. Materials and methods

2.1. In vitro morphogenesis of Digitalis purpurea

Digitalis purpurea cv. Berggold shoot cultures were initiated from *in vitro* germinated seeds, using only one line to avoid heterogeneity. *In vitro* plants, considered as mother or control plants were cultured on solid medium as previously described (Pérez-Alonso et al., 2009). Briefly, shoots were multiplied inducing multiple shoots from axillary buds in flasks containing MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg l^{-1} thiamine HCl, 4.4 μM 6-benzylaminopurine (6-BAP), 0.57 μM indole acetic acid (IAA), 100 mg l^{-1} *myo*-inositol, 30 g l^{-1} sucrose and 3.0 g l^{-1} Gelrite (Duchefa, Netherlands). The pH was adjusted to 5.8 with 0.5N KOH or 0.5N HCl before autoclaving at 1.1 kg cm^{-2} and 121 °C for 20 min. The cultures were incubated in a growth chamber at 27 ± 2 °C under a 16 h photoperiod from cool white fluorescent lamps at a photosynthetic photon flux density of 70 μmol $m^{-2}s^{-1}$. Mother plants were sub-cultured every 28 days as mentioned above.

For shoot induction, leaf segments (1.0 cm^2, adaxial surface to the medium) from *in vitro* plants (fourth-seventh subculture) were cultured on basal medium containing MS salts supplemented with 4.0 mg l^{-1} thiamine HCl, 100 mg l^{-1} *myo*-inositol, 30 g l^{-1} sucrose and 3.0 g l^{-1} Gelrite (Duchefa, Netherlands).

The effect of naphthaleneacetic acid (NAA) 0, 0.54 or 2.7 μM combined with 6-BAP 0, 4.4, 13.2 or 22.0 μM was tested on MS basal medium. The pH was adjusted to 5.8 with 0.5 N KOH or 0.5 N HCl prior to autoclaving at 1.1 kg cm^{-2} and 121 °C for 20 min. This medium was called Shoots Induction Medium. Culture conditions were the same as mentioned above.

Evaluation of the percentage of leaf segments that produces adventitious roots or shoots, and the number of regenerated shoots per leaf segment were recorded after six weeks. Leaf segments with clearly differentiated shoots and leaves of approximately 1.0–2.0 cm in length were scored as leaf segments with shoots. The morphology of the formed shoots was also evaluated. Then, developed shoots were transferred to jar flasks containing 30 ml of MS medium supplemented with 1.0 mg l^{-1} thiamine HCl, 4,4 μM 6-BAP, 0.57 μM IAA, 100 mg l^{-1} *myo*-inositol, 30 g l^{-1} sucrose and 3.0 g l^{-1} Gelrite, pH 5.8; and subcultured every four weeks to the same fresh medium. Culture conditions were the same as mentioned above.

Ten replicates were done for each treatment (10 jar flasks with five explants each = 50 explants). The experiment was repeated four times.

2.2. Analysis of genetic stability using RAPD

Genetic homogeneity between the mother plant and selected de novo regenerated plantlets was assessed using RAPD (Random Amplified Polymorphic DNA). Twelve plantlets obtained from the best combination of growth regulators were randomly selected for this analysis after three subcultures. DNA was isolated from 100 mg of leaves of regenerated plants and the mother plant using the protocol described by Khayat et al. (2004). Genomic DNA integrity was analyzed through electrophoresis in 0.8% (w/v) of agarose gel 1X Tris–Borate–EDTA (TBE 1 X: 0.89 M Tris-HCl, 0.02 M EDTA and 0.89 M boric acid) and purity was analyzed in a Biophotometer (Eppendorf, Germany). Purified DNA was kept at −20 °C for further analysis.

A total of eight arbitrary decamer primers (Center of Genetic Engineering and Biotechnology, Havana, Cuba) and some combinations were tested to amplify fragments from genomic DNA of mother and de novo regenerated plants. Only primer combinations that produce distinct easily scorable amplification profiles were selected (Table 2). Amplification was performed using genomic DNA of each plant as a target. PCR was carried out in 30 μl total volume containing 100 ng of template DNA, 200 mM dNTPs, 1.5 mM $MgCl_2$, 1X TopTaq polymerase reaction buffer (QIAGEN, Germany), 1 unit of TopTaq DNA polymerase (QIAGEN, Germany) and 0.5 μM of primers. Amplification reaction was carried out using a MasterCycler ep Gradient (Ependorff, Germany) with an initial denaturation of DNA at 94 °C for 4 min, followed by 35 cycles consisting of 30 s denaturation at 94 °C, 30 s annealing at 30 °C and 2 min extension at 72 °C. These cycles were followed by a final ex-

tension of 15 min at 72 °C and hold temperature of 4 °C. Amplified fragments were analyzed by electrophoresis at 80 V for 2 h on 1.5% agarose gel in 1X TBE buffer, followed by staining in ethidium bromide (5 μg ml^{-1}).

After electrophoresis and staining, amplified bands were photographed under ultra-violet light using a Gel Documentation & Analysis System (WD-9413A). All the PCR reactions were performed at least twice to check the reproducibility.

2.3. Pharmacognostic analysis

2.3.1. Microscopic analysis and numeric indexes determination

Samples of *Digitalis purpurea* leaf powder were produced from three randomly selected plants previously regenerated *via* direct organogenesis. Control was defined as one sample of leaf powder from a mother plant. Leaves from *in vitro* cultured plantlets were rinsed with distilled water and dried in an oven at 50 °C until the weight was constant. Then, dried material was powdered with a mortar and pestle. The resulting powder was passed through a 500 μm sieve to obtain a fine dust. Thereafter, powder was examined under a microscope using chloral hydrate solution (80 g of chloral hydrate in 20 ml of water). In addition, loss on drying, total ash and acid-insoluble ash were determined according to the British Pharmacopoeia (BP) (2013 sections 2.2.32; 2.4.16; 2.8.1) and Evans (1996). Data are means from five samples of de novo regenerated and mother plants.

2.3.2. Kedde test

A test solution was prepared for a colorimetric test, according British pharmacopoeia (2013) and Evans (1996). Briefly, 20 ml of ethanol (50%, v/v) and 10 ml of lead acetate solution (9.5%, m/v) was added to 1.0 g of powdered plant material. Then, the solution was boiled for 2 min, cooled down at room temperature and centrifuged at 5000*g*. Then, glycosides were extracted with 2 vols of chloroform. Thereafter, excess of water was dried over anhydrous sodium sulfate and filtered by a filter paper (Whatman 1). Chloroform solution was then evaporated on a water-bath. Afterward, 1.0 ml of the concentrated solution was mixed with 2.0 ml of a mixture of dinitrobenzoic acid solution with ethanol 96% (2.0% m/v) and 1.0 ml of 1.0 M sodium hydroxide. After 5 min, the color change was observed.

2.3.3. Cardenolide content

In order to determine the content of cardenolides and compare de novo regenerated plants with mother plants, samples of leaf powder were analyzed. Samples of 1.5 g of powdered plant material were extracted with 15 ml ethanol (70%) in an ultrasonic bath at 70 °C for 15 min using a method previously described by Wichtl et al. (1982) with modifications introduced by Pérez-Alonso et al. (2009). The residue obtained after extraction and rotaevaporation was dissolved in 1.0 ml of ethanol for High Performance Liquid Chromatography (HPLC) analysis. Ten microliters of this solution were injected in an Agilent 1100 HPLC system equipped with a diode array detector and an Inertsil ODS-3 column (150 × 4.6 mm; 5 μm). A mixture of acetonitrile/water (25/75; v/v) was used as eluent at a flow rate of 1.5 ml min^{-1}. All measurements were carried out at 40 °C and glycosides were detected at 220 nm. Digitoxin and digoxin were identified based on their retention time and the comparison of their UV spectra with those of authentic standards obtained from a commercial source (SIGMA).

2.4. Statistical analyses

A completely randomized design was used for all treatments. Statistical analyses were performed using computer software SPSS package for Windows ver. 21. Data were analyzed by non-parametric Kruskal-Wallis test. To distinguish between comparisons a post hoc Mann-Whitney test was performed. Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. In vitro morphogenesis of Digitalis purpurea

After seven days of culture leaf segments cultured on MS medium supplemented with combinations of NAA and 6-BAP (Fig. 1A) or 6-BAP alone produced visible zones of high mitotic activity. These regions produce meristemoids, which are defined as a cluster of isodiametric cells within a meristem or cultured tissue, with the potential to develop an entire plant (George and Debergh, 2008). On the other hand, the explants cultured on media containing NAA alone showed adventitious rooting (Fig. 1B, Table 1). However, leaf explants cultured on a medium without growth regulators (control) did not show any positive response. After 10 days of culture, some sectors of the leaf segments turned brown in the control treatment, which became unviable for regeneration (Fig. 1C).

After 20 days of *in vitro* culture the first shoots were observed through a stereoscope. Because of the combination of NAA and 6-BAP we appreciated some morphogenetic changes (Fig. 1D–F). After six weeks, about 98.5% of the leaf segments directly formed shoots on MS medium containing 0.54 μM NAA and 13.2 μM 6-BAP, while with 6-BAP alone, the frequency of regeneration was very poor (15–25% of leaf segments with shoots). Our results also showed that this combination increased the number of de novo regenerated shoots per leaf segment (18.9) with significant differences with the rest of the treatments (Table 1).

The organogenesis process is considered very complex, involving several internal and external factors. This morphogenetic process consists of several stages, including dedifferentiation of the target tissue and initiation of the various developmental stages which culminate in the production of a developed shoot (Joy and Thorpe, 1999).

Organogenesis had been achieved in different species of *Digitalis* genus *e.g. D. thapsi* (Cacho et al., 1991), *D. trojana* (Çördük and Aki, 2010), *D. davisiana* (Gurel et al., 2011), *D. lamarkii* (Verma et al., 2011a,b), *D. nervosa* (Karimi and Kazemitabar, 2013), *D. purpurea* (Patil et al., 2013; Li et al., 2014) and recently in *D. cariensis* (Mohammed et al., 2015). The success of regeneration depends on the type and concentration of plant growth regulator. In most cases the combination of auxins and cytokinins induces morphogenesis better than auxin or cytokinin alone (Zhao et al., 2014). Previous studies have shown a positive response of several *Digitalis* species to 6-BAP and NAA combination (Pérez-Bermúdez et al., 1984; Cacho et al., 1991). The same growth regulators combination was also successful for this morphogenetic process in other closely related species such as *D. trojana* (Çördük and Aki 2010) and *D. nervosa* (Karimi and Kazemitabar, 2013). Çördük and Aki (2010) described an efficient protocol for *in vitro* propagation *via* direct adventitious shoot organogenesis from leaves (32% of explants forming shoots with 28 shoots per explant). While, Karimi and Kazemitabar (2013) reached 93.7% of shoot organogenesis with 6–9 axillary shoots per hypocotyl as the best explant in *D. nervosa*. It has been shown that addition of a low amount of NAA in combination with 6-BAP increased significantly the endogenous concentration of total isoprenoid-type cytokinins in *Eucomis zambesiaca* when it was com-

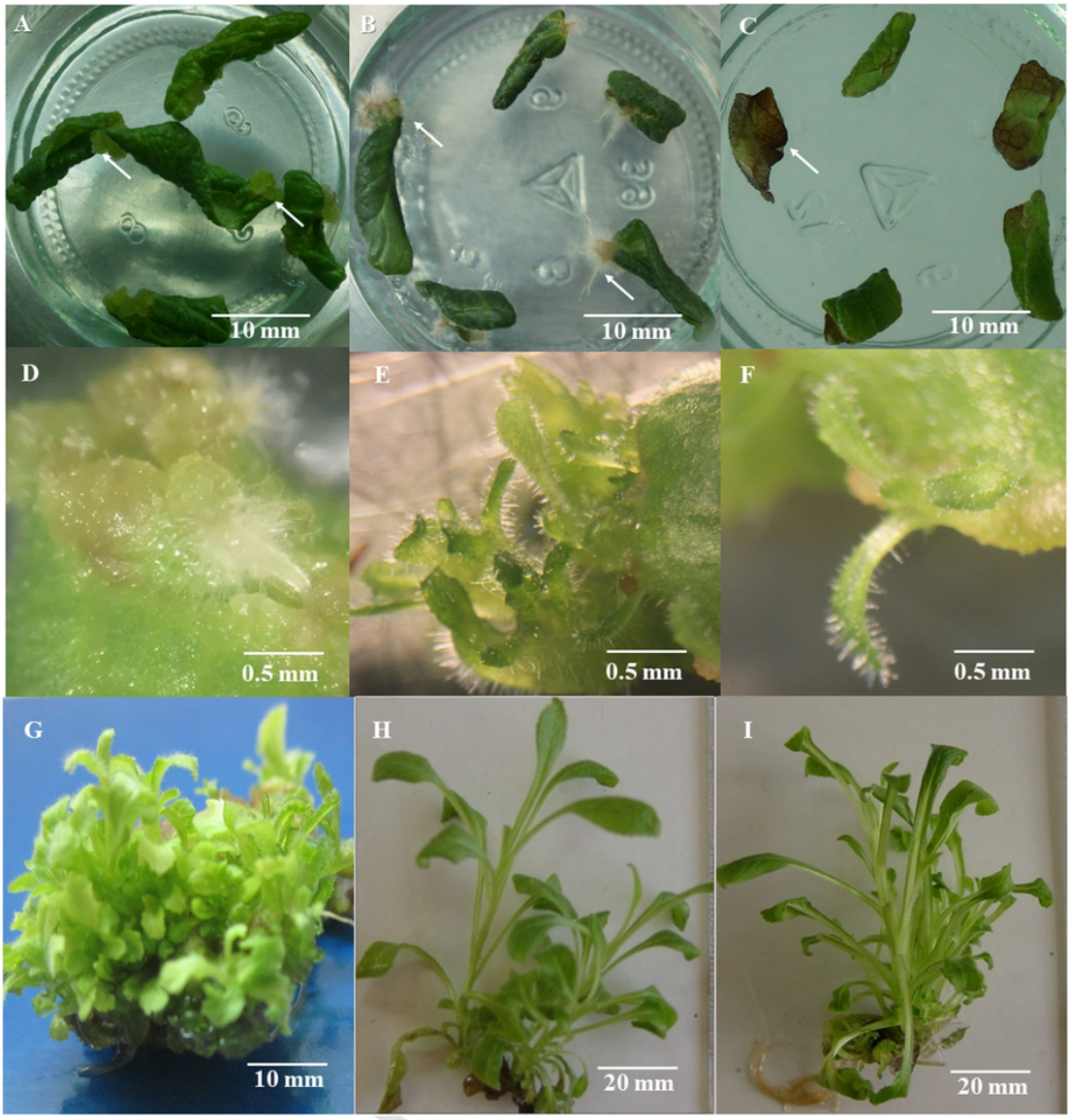

Fig. 1. Morphogenetic process from *in vitro*-derived leaf explants of *Digitalis purpurea* L. after 15 (A–C), 20 (D) and 25 (E–F) days respectively in culture under different conditions. A: Meristemoid formation on MS medium supplemented with 0.54 μM NAA and 13.2 μM 6-BAP, B: Adventitious rooting on MS medium containing 2.7 μM NAA, C: MS medium without growth regulators produced necrotic damage on leaves, D-E: Shoot formation on MS supplemented with 0.54 μM NAA and 13.2 μM 6-BAP, F: simple, alternate leaves, in basal rosette shape disposition, G: Morphological aspect of de novo regenerated plants after three subcultures, shoots during multiplication phase spontaneously developed roots in MS medium, H: De novo regenerated plants showed no morphological changes compared to (I) mother plant.

pared with 6-BAP alone (Aremu et al., 2016). This increase was remarkably higher on *cis*-zeatin and isopentenyladenine. However, in *Eucomis autumnalis* subspecies *autumnalis* a significant increase in the endogenous concentration of aromatic-type cytokinins was observed when NAA was added to the regeneration medium (Aremu et al., 2016). Notwithstanding the contrasting results, in both species the combination of auxin and cytokinin resulted in higher response on shoot number.

In *D. purpurea*, Patil et al. (2013) described a protocol for *in vitro* propagation *via* direct organogenesis using several explants including leaf segments. These authors reported the highest response (85.7%, 12.7 shoots per explant) from nodal explants cultured on MS medium supplemented with 7.5 μM 6-BAP. However, leaf explants showed the lowest response (57.1%, 7.2 shoots per explant), and this response was even lower when auxin was added to the medium. The differences with our results could be due to the use of leaves from 21 days old seedlings as initial explants by Patil et al. (2013). In fact, in *Arabidopsis thaliana* and *Nicotiana tabacum,* Ljung et al. (2001) found that IAA can be synthetized in seedling leaves. These authors observed that very high levels of IAA in both species were temporally correlated with the high division rates and the beginning of vascular differentiation. Moreover, there is evidence that IAA could be synthetized in the chloroplast and in other parts of the plants and thereafter transported to young leaves and storage in the chloroplasts (Dong et al., 2014). Differences among shoot regeneration capacity have been associated to dissimilar balance of endogenous plant growth regulators in different types of explants in *Digitalis* species and other medicinal plants (Gurel et al., 2011; Moharami et al., 2014; Mohammed et al., 2015). In addition, exogenously applied growth regulators affected their levels altering the biosynthesis, distribution and some aspects of cell growth and differentiation process and finally influencing *in vitro* regeneration (Amoo and Van Staden, 2013; Ćosić et al., 2015).

Usually, optimization of *in vitro* regeneration protocols requires variation of the composition and the ratio of growth regulators empiri-

Table 1
Morphogenetic responses in *Digitalis purpurea* L. leaf explants on MS basal medium containing NAA and 6-BAP alone or in combination at 42 days of culture.

NAA + 6-BAP (μM)	% of leaf segments developing adventitious roots	% of leaf segments with shoots	No. of regenerated shoots/leaf segment
0.00 + 0.00	0.0 b	0.0 h	0.0 h
0.54 + 0.00	80.0 a	0.0 h	0.0 h
2.70 + 0.00	84.0 a	0.0 h	0.0 h
0.00 + 4.40	0.0 b	20.3 fg	0.4 fg
0.00 + 13.20	0.0 b	25.0 f	0.3 g
0.00 + 22.00	0.0 b	15.0 g	0.7 f
0.54 + 4.40	4.0 b	81.5 b	10.2 b
0.54 + 13.20	0.0 b	98.5 a	18.9 a
0.54 + 22.00	4.0 b	52.0 e	8.2 c
2.70 + 4.40	4.0 b	63.0 cd	3.8 e
2.70 + 13.20	2.0 b	69.5 c	8.0 cd
2.70 + 22.00	0.0 b	53.5 de	7.1 d

Data are means from four independent experiments, each with 50 explants (n = 40 for percentage and n = 200 for number of de novo regenerated shoots). Values followed by different letters per column for each variable are significantly different ($P \leq 0.05$) based on Kruskal-Wallis/Mann-Whitney Tests.

cally. However, in these experiments it is important to consider the fundamental role played by the balance between the different plant hormones in the morphogenetic development in these processes (Guo et al., 2017).

Li et al. (2014) described the second regeneration protocol through direct organogenesis in *D. purpurea*. They described 100% adventitious shoot regeneration from mature leaf segments cultivated on MS medium containing 4.5 μM TDZ and 0.54 μM NAA. However, data about the number of shoots regenerated per explant was not provided. Although these two reports are available for direct organogenesis in *D. purpurea*, the authors did not refer to genetic stability of regenerated plants, nor to analysis of leaf powder quality of *in vitro* plantlets.

De novo regenerated shoots from the best treatment spontaneously developed roots during the multiplication phase on MS medium supplemented with 4.4 μM 6-BAP + 0.57 μM IAA (Fig. 1G). The de novo regenerated plantlets from the best treatment (Fig. 1H) showed no significant morphological differences compared to mother plants (Fig. 1I). The above media can induce the formation of more than 47 leaves per de novo regenerated plant, which were ovate lanceolate or broadly ovate with an average length of 9.3 cm.

In contrast with indirect organogenesis, which has been described for *D. purpurea* (Pérez-Alonso et al., 2014a,b), the method proposed in this work reduces the time of culture and avoids a callus phase. In addition, a callus phase has been described as a possible factor increasing somaclonal variation in the plantlets regenerated *via* indirect organogenesis (Wang et al., 2012).

The results described above offer an efficient and practical method for clonal propagation of *D. purpurea*, which is a prerequisite for several in biotechnological and genetic engineering approaches in breeding programs.

3.2. Analysis of genetic stability using RAPD

RAPD analysis did not show any detectable genetic variation in the de novo regenerated plants compared with the mother plant (Table 2, Fig. 2). This analysis produced 126 unambiguous bands from eight combinations of four primers (Table 2). The number of bands produced per primer combination ranged from 12 to 23 (Table 2), with an average of 15.75 bands/primer combination. A total of 1638 monomorphic bands (126 bands ×13 analyzed plants) were amplified in this analysis from a mother plant and 12 selected clones regenerated through the protocol described above. Then, it can be concluded that direct organogenesis of *D. purpurea* could be used as fast micropropagation tool with minimal risk of genetic instability.

RAPD analysis has been used in *Digitalis* to test genetic stability after *in vitro* culture procedures, but in both cases in *D. obscura* (Gavidia et al., 1996; Sales et al., 2001). In the first case, Gavidia et al. (1996) did not find any differences between long term *in vitro* cultured plants and mother plants. On the other hand, Sales et al. (2001) found up to 15.1% of polymorphism after 16 subcultures. Furthermore, differences in metabolite content were detected in *D. mariana* ssp. *heywoodii* plants regenerated from callus or shoots (Kreis et al., 2015). These differences were dependent on the time of the morphogenetic process, but genetic analysis to confirm the origin of this variation was not performed (Kreis et al., 2015).

In recent years, RAPD analyses have been used by many authors to detect somaclonal variability/homogeneity of medicinal plants regenerated from *in vitro* culture and to corroborate if they are identical to the original plant; *e.g. Withania somnifera* L. (Nayak et al., 2013), *Terminalia bellerica* (Gaertn) Roxb (Dangi et al., 2014), *Ceropegia evansii* McCann (Chavan et al., 2015a) and *Salacia chinensis* L. (Chavan et al., 2015b).

RAPD markers have gained considerable importance in genetic fidelity assessment due to many desirable characteristics like simplicity, efficiency, cost-effectiveness and being a method that can be performed without the use of radioactivity (Haque and Ghosh, 2013).

The results obtained suggest that direct organogenesis in *D. purpurea* from leaf explants carries minimal risk of generating somaclonal variation.

Table 2
The decanucleotide sequences of primers, total number and size range of amplified fragments per primer (combination) in random amplified polymorphic DNA (RAPD) analysis used for testing the genetic stability of *Digitalis purpurea* obtained *via* direct organogenesis.

Primer code	Primer sequence (5′-3′)	Number of scorable/monomorphic bands	Size range of amplification products (bp)
2	5′-GAGGGACCTC-3′	15/15	400–3000
3	5′-GGGCTATGCC-3′	20/20	200–3000
5	5′-GATGACCGCC-3′	12/12	400–1700
6	5′-ACCGCGAAGG-3′	15/15	400–1800
2–5	–	12/12	300–1500
2–6	–	15/15	200–1800
3–5	–	23/23	290–2000
3–6	–	14/14	300–2000
Total		126	

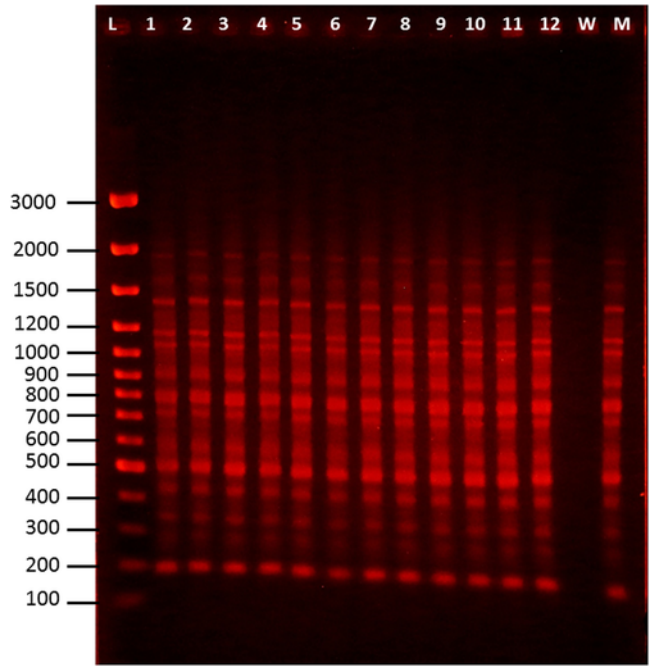

Fig. 2. Gel electrophoresis of a RAPD amplification profile obtained with primer 2 of *Digitalis purpurea* mother plant (M) and twelve (1–12) randomly selected plants regenerated by direct shoot organogenesis. Lane L: GeneRuler 100 bp Plus DNA Ladder (Thermo Scientifc).

3.3. Pharmacognostic analysis

Leaf powder analysis in a microscope at different magnifications showed all the anatomical structures described in BP for this species such as stomata and trichomes (Fig. 3A–D). The powder shows fragments of the upper epidermis with cells with a smooth cuticle and anticlinal walls. Trichomes are of two types: uniseriate, covering trichomes with blunt apex, usually consisting of 3–5 cells and glandular trichomes with a unicellular sometimes a multicellular, uniseriate stalk and a unicellular head or bicellular head. No differences were observed between mother plants and regenerants. That is a phenotypic indicator of the homogeneity and quality in the produced powder from *in vitro* regenerated plants.

The results for numeric indexes, which are quality parameters, were in the range of the values accepted in BP (Table 3) without statistical differences between de novo regenerated and mother plants. The parameter 'loss on drying' is related to the humidity of the environment and may affect the quality of herbal drugs during storage. A high content of water may favor the growth of microorganisms (*e.g.* fungi which produce mycotoxins) affecting the quality of the powder and reduce or inactivate the therapeutic activity of the pharmaceutical product (Duţu, 2012).

The high content of water is related with hyperhydricity, a phenomenon described as physiological disorder of *in vitro* grown plantlets (Debergh et al., 1992). Lapeña et al. (1992) described that hyperhydricity drastically altered the cardenolide accumulation in *in vitro* grown plants of *D. obscura*. This negative effect was also evident in *D. minor* (Sales et al., 2002). Water content may activate enzymatic systems such as specific hydrolases, which may degrade primary cardenolide glycosides to secondary cardenolide glycosides. The latter compounds have less activity (Duţu, 2012). Low moisture suggests better

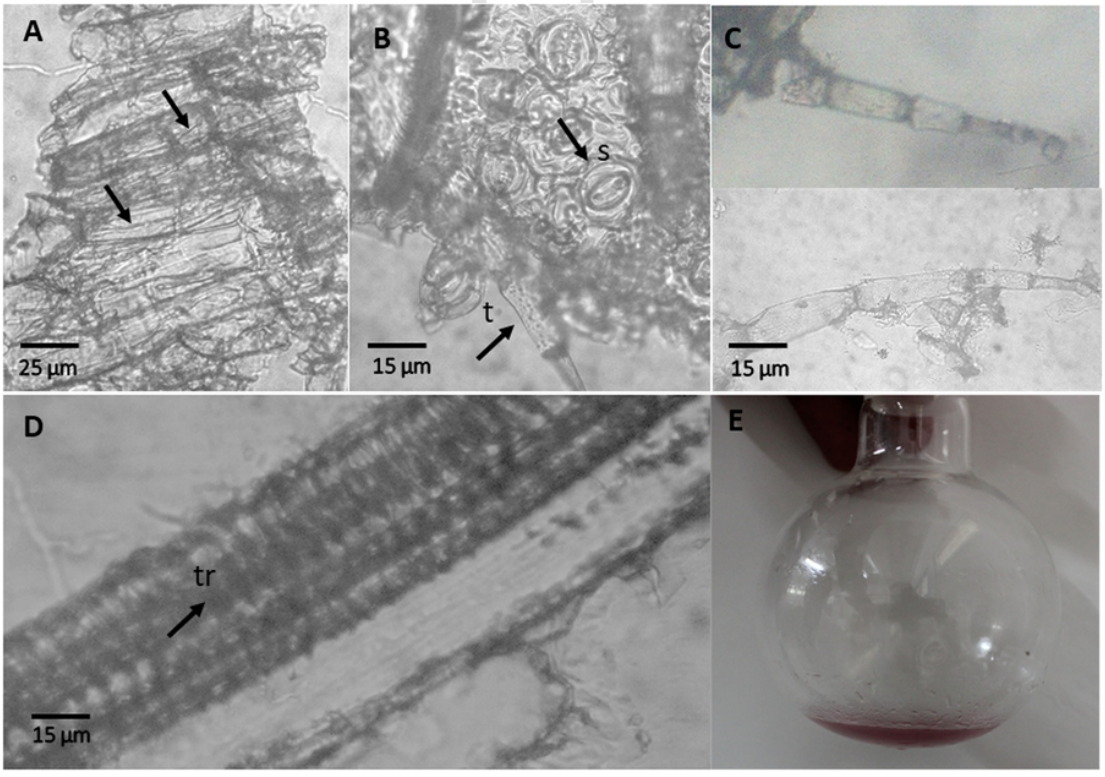

Fig. 3. Microscopic examination of leaf powder of *Digitalis purpurea* L. A: Epidermis, B: stomata (s) and trichomes (t), C: glandular trichomes with multicellular (four and five cells), uniseriate stalk and a unicellular head, D: simple pitted vessels and tracheids (tr). E: Test of Kedde, a qualitative chemical analysis.

Table 3
Numeric index result of *in vitro Digitalis purpurea* L. regenerated plants *via* direct organogenesis compared to mother plants (control).

Leaf powder source	Loss on drying (%)	Total ash (%)	Ash insoluble in hydrochloric acid (%)
Mother plant	4.66 a	7.05 a	1.54 a
De novo regenerated plant	4.49 a	6.44 a	2.06 a
Maximum value accepted by PB	6.0	12.0	5.0

Mother plants were initiated from *in vitro* germinated seeds using one line to avoid heterogeneity Data are means from 10 replicates. Values followed by same letters per column for each variable are equal ($P > 0.05$) based on Kruskal-Wallis Test.

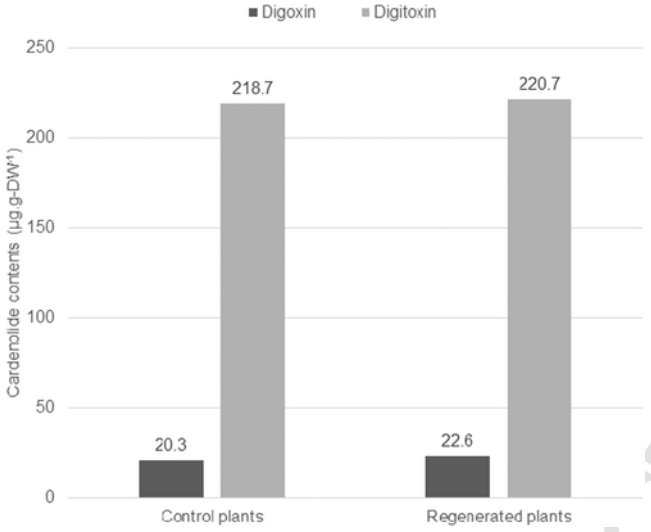

Fig. 4. Content of cardenolides in *Digitalis purpurea* plants regenerated *via* direct organogenesis compared to mother plants. Mother plants were initiated from *in vitro* germinated seeds using one line to avoid heterogeneity. Bars with same letters for each parameter are equal based on a Kruskal-Wallis test.

stability against degradation of product. Nevertheless, plants regenerated through our system do not have symptoms of hyperhydricity, condition that benefit the quality of leaf powder.

Total ash and ash insoluble in hydrochloric acid express the content of metal ions (mineral compounds) of a vegetal drug. Total ash includes physiological ash, which is derived from the plant tissue itself and non-physiological ash, which is often from environmental contaminations. These results were expected for *in vitro* regenerated plants which came from innocuous and sterile medium.

The test of Kedde is a qualitative chemical analysis. This test was positive for mother and de novo regenerated plants. After 5 min a reddish-violet color was developed (Fig. 3E). These results indicate the presence of cardenolide glycosides (Duţu, 2012).

The quality of *D. purpurea* leaf powder according BP, to our knowledge, has not been described previously for *in vitro* regenerated plants *via* direct organogenesis.

HPLC analysis revealed that the plants regenerated *via* direct organogenesis had an appropriate amount of digoxin and digitoxin (Fig. 4), not significantly different from that of mother plants. Therefore, plants regenerated using the protocol described above could be used as a raw material for cardenolide production. This production in *Digitalis* plants, has proved to be affected by environmental factors, developmental stage as well as method of propagation (Sales et al., 2011). Knowledge of the biosynthetic pathway is limited and there is no report on the interaction of plant growth regulators and production of these compounds. In the present study, both de novo regenerated and mother plants were grown on a medium with 6-BAP. According to Karimi and Kazemitabar (2013) in regenerated shoots of *D. nervosa via* direct organogenesis, 6-BAP increased content of cardiotonic glycosides, aspect that can be explain no differences on cardenolide content between regenerated plants *via* direct organogenesis and mother plants.

Patil et al. (2013) found a higher content of cardenolides than described in the present study, however they fortified the medium with progesterone (200–300 mg l^{-1}), a precursor associated with the biosynthesis of cardiotonic glycosides; this is, however, an expensive strategy for commercial production.

The protocol described here could be successfully used for mass propagation of *D. purpurea* avoiding the disadvantages of cultivation in the field. As such, this technique could be an important source of raw material for cardenolide production. Moreover, this protocol could be associated with other strategies such as temporary immersion system for a large-scale cultivation, which will help to achieve better results for pharmacological industry confirming the practical applicability of plant tissue culture.

On the other hand, the organogenesis protocol presented here opens possibilities for genetic transformation and metabolic engineering studies in order to increase cardenolide content in *in vitro* plants and to avoid the excessive use of natural populations.

Conflict of interest

The authors jointly declare that there are no conflicts of interests

Acknowledgements

The authors wish to thank the support of the Cuban National Program of Exact Science from the Ministry of Higher Education through the project P223LH001-037 and the Joint PhD between Vrije Universiteit Brussel and Cuban Universities.

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